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THE DESCRIPTION OF A BLOOD GROUP SYSTEM IN DEER MICE

(PEROMYSCUS MANICULATUS)

by



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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled The Description of a Blood Group System in Deer Mice (*Peromyscus maniculatus*) submitted by Elizabeth A. Savage, in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

By means of isoimmune antisera produced in deer mice, *Peromyscus maniculatus*, a simple two allele blood group system in that species was expanded into a complex blood group system with at least four and possibly six alleles. Serological results indicated that at least two of the alleles were determining more than one antigenic specificity.

Breeding data showed that females of a certain heterozygous phenotype were segregating a disproportionate number of alleles of one type to their offspring. At this time there is no obvious explanation for this occurrence.

Blood samples from several species of *Peromyscus* were tested with the antisera and, with the exception of *P. polionotus*, all gave negative results. In addition, blood samples from various populations of deer mice were typed and the results showed that although allele frequencies seemed consistent from generation to generation within populations, the allele frequencies varied between populations.

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INTRODUCTION

Immunological studies have revealed that erythrocytes possess chemical structures on their surfaces which are antigenic to other individuals not possessing the equivalent antigens. The immunological response results in the formation of specific antibodies which react with the foreign red blood cells. The reaction *in vivo* is characterized by the lysis of the cells whereas the manifestation of the antibody-antigen reaction *in vitro* may be agglutination or lysis of the cells or the inhibition of the antibody by group specific substances (Dunsford and Bowley, 1967).

The substances on the red blood cell which react with specific antibodies are called blood group antigens and their existence is determined by the presence and action of genes. Sometimes several variant antigens are produced by different alleles of one gene locus in a species and collectively these antigens constitute a blood group system.

The first blood group system was discovered by Landsteiner in 1900 with the discovery of the ABO system in man. His discovery was facilitated by the fact that man has naturally occurring antibodies which react with two antigens in that system, a phenomenon which does not occur for all systems nor in all species (Wiener, 1943). The need to match blood for blood transfusions and the discovery that blood group incompatibilities were responsible for hemolytic disease of the newborn (Levine and Stetson, 1939) made the study of blood groups very important and necessary in clinical medicine, and provided the impetus for a great deal of research into human blood group systems (Race and Sanger, 1968).

Initially the use of animals was confined to research into problems

of human blood group systems. Each advance made in the knowledge of human blood groups led to its experimental extension in laboratory animals (Cohen, 1962), e.g. the discovery of the ABO system provided the stimulus for the discovery of erythrocytic antigens in the rabbit (Hulot and Ramond, 1901). However, interest in animal blood groups grew when it became apparent that blood group antigens were characters which could be accurately identified, which were developed from the action of specific genes, with little or no modification by environment, and which varied considerably between individuals within species or strains. The blood group genes in animals came to be thought of by some breeders and research workers as marker genes which might show association with production traits and this led to intensive study on blood groups primarily in domestic animals. Although experiments with a few species have indicated that some blood groups seem correlated with production, e.g. the B system in chickens (Briles and Allen, 1961; Allen, 1962; and Allen and Gilmour, 1962), it seems likely from the point of view of commercial breeding, that results can be achieved more readily by the use of conventional selection techniques than by blood typing (McDermid, 1966; Neimann, Sorenson and Robertson, 1961). Blood groups, however, have proven useful to breeders in solving problems of parentage, misidentification, and mispedigreeing.

In addition to the applied use of blood groups, both in animals and man, considerable emphasis has been placed in recent years on studies aimed at clarifying the nature of the genetic mechanism involved in the determination and control of blood group antigens themselves (Watkins, 1966).

As outlined by Gilmour (1970) blood group systems can be roughly divided into two classes, depending upon the nature of the antigens involved.

A blood group system may be of a simple type in which few alleles, often only two, occur at a locus and these control the inheritance of the same number of simple antigens. The most elementary concept of genetic control for this type of system is the "one to one" theory in which each antigen results from the action of a single allele and consists of one substance which is capable of stimulating the formation of a single specific antibody. The antigens of a simple system would thus rarely exhibit cross-reactivity.

The second class of systems is more complicated and involves the occurrence of complex antigens. A complex antigen may be thought of as made up of an assortment of sub-antigens or antigenic factors, some of which are the same as, and others different from, the factors making up other complex antigens within the same system (Gilmour, 1970). Each complex antigen is inherited as a unit which is sometimes referred to as a "phenogroup," and the genetic control of such units has long been a subject for much speculation.

One of the best known complex systems is the Rh blood group system in man. Two schools of thought emerged regarding its genetic control and these serve to illustrate the nature of the controversy. Fisher's theory (Race and Sanger, 1968) was similar to the "one to one" theory in that he proposed that the antigenic complexes were determined by a series of closely linked genes, the alleles of which would determine one antigenic determinant or factor. Wiener (Wiener and Wexler, 1958), on the other hand, proposed a single Rh locus with multiple alleles, each capable of determining a number of Rh antigenic determinants.

The differences in concept are not likely to be resolved until more information has been accumulated, especially as to the biochemical nature

of the antigens (Shreffler, 1967). If the antigens are the protein products of the blood group locus, that is they are structural proteins in the cellular membrane, it is possible that a series of mutational events within a gene could give rise to multiple antigenic determinants within a single polypeptide chain, and thereby support Wiener's theory. However, if the antigens are carbohydrate, such as the ABO and Lewis antigens (Watkins, 1966), then the products of blood group loci would be enzymes concerned with the attachment of sugar groups to polysaccharide chains or to protein or lipid carriers. It is difficult to imagine how the enzyme products of alleles of a single gene could synthesize multiple differences in carbohydrate molecular conformation.

Most animals which have been extensively studied have been shown to have at least one blood group system which exhibits complexity similar to the Rh system in man, e.g. the B system in chickens (Briles, Allen and Millen, 1957), the B system in cattle (Stormont, Owen and Irwin, 1951), the E system in pigs (Anderson, 1962), and the H-2 system in mice (Amos, 1962). These animals have also been shown to have simple systems as well; however it is possible that some of these simple systems will also prove to be complex in the future. Race and Sanger (1959) point out that the simplest blood group systems tend to be those most recently discovered and, with time, they are usually shown to be more complicated than originally described. A good example of this is the MN blood group system in man. It was originally described as a two allele, three phenotype blood group system in 1928 (Landsteiner and Levine, 1928) and was thought of as such for 20 years until the discovery of the S blood factor (Walsh and Montgomery, 1947). Since then it has been expanded into a complex system involving possibly 30 alleles.

This thesis is concerned with the characterization of a blood group system in the deer mouse (*Peromyscus maniculatus*) with the intended purpose that this could provide useful genetic markers for population studies.

Deer mice are especially suitable for this type of genetic study because they are easily captured, breed well in captivity, are multiparous, and have a relatively short generation interval. In addition, numerous species within this genus are widely distributed throughout North America.

Several population studies have used deer mice (Rasmussen, 1964; Canham, 1969; Birdsall, 1971). Lewontin (1967) has pointed out that *Peromyscus* is an ideal animal for population studies because laboratory breeding is possible, many loci can be characterized in each individual and abundant natural populations are available for manipulation.

Blood group loci are useful in these types of studies as convenient genetic markers. In addition, because of the variety of alleles occurring at blood group loci, it is possible that the study of the frequencies of blood group alleles within species and subspecies may prove useful in establishing evolutionary relationships within genera (Stormont, 1962).

The initial work done on blood group antigens in deer mice was done by Moody (1941) who demonstrated the existence of different cellular antigenic components between the species *P. maniculatus* and *P. leucopus*, using immune rabbit sera. Cotterman (1944) also showed that erythrocytic antigen variation occurred between various *Peromyscus* species, using normal human group B sera. The first work done on erythrocytic antigen differences within a species of the genus *Peromyscus* was done by Moody (1948) who demonstrated antigenic differences between populations of *P. maniculatus*. The first known blood group system in the deer mouse was described by Rasmussen (1961).

Rasmussen detected two antigenic types which appeared as unitary genetic characteristics inherited as a two allele, one locus system. He designated the alleles as Pm^A and Pm^B and the corresponding antigens as A and B. Mice could be typed as A, B, or AB. He detected these antigens with heteroimmune rabbit sera which had been adsorbed with cells from individual deer mice. Later he found that he could get parallel results using isoimmune mouse sera. He detected these antigens in two subspecies of *P. maniculatus* and in addition detected the A antigen in *P. polionotus*, a member of a taxon, the "*maniculatus* group." Rasmussen also tested *P. leucopus* and various laboratory strains of *Mus musculus* with negative results.

Birdsall in 1967 (unpublished) used the isoimmunization methods outlined by Rasmussen and was able to demonstrate erythrocyte antigenic variation among individual deer mice (see Table I). He, however, did not use these results to hypothesize a blood group system.

The following study did use his results and reagents in initial stages of the isoimmunization program to hypothesize a blood group system. The blood group system which was finally established proved to be concerned with the Pm locus described by Rasmussen. During the course of the work it was discovered that the Pm locus is in fact a complex locus with at least four and possibly six alleles. During this study many species of *Peromyscus* were tested and, with the exception of *P. polionotus*, the results indicate that the antigens determined by the Pm locus are confined to *P. maniculatus*. The frequency of the alleles at this locus, however, vary considerably among the subspecies of *P. maniculatus*.

These observations indicate that further analysis of this locus could prove valuable in describing the evolutionary relationships within

Table I. Reactions of Birdsall's isoimmune antisera with erythrocytes of individual deer mice

Deermouse erythrocytes	7	34	60b	60c	60e ₁	76	Isoimmune antisera 60a	76d	60c ₁	76c	76e	96c	96d	96e
7	-	-	+	+	+	+	w	-	-	-	-	-	-	-
34	-	-	+	+	+	-	w	-	-	-	-	-	-	-
96d	-	-	+	+	+	+	w	-	-	-	-	-	-	-
76c	-	-	+	+	-	+	w	-	-	-	-	-	-	-
76d	-	+	+	+	+	+	w	-	-	-	-	-	-	-
96e	-	+	+	+	+	+	w	-	-	-	-	-	-	-
60c	w	-	+	w	+	w	w	-	-	-	-	-	-	-
76e	w	+	+	+	+	+	w	-	-	-	-	-	-	-
60a	+	+	-	+	-	-	w	-	-	-	-	-	-	-
60c	w	+	-	-	-	-	w	-	-	-	-	-	-	-
96c	w	+	-	-	-	-	w	+	-	-	-	-	-	-
60b	w	-	-	-	-	-	w	-	-	-	-	-	-	-
76	w	-	-	-	-	-	w	-	-	-	-	-	-	-
60e	-	-	-	-	-	-	w	+	-	-	-	-	-	-
Suggested classification	Anti-A			Anti-B			non- specific	unknown	non-reactive					

+ = positive agglutination of red blood cells
 - = no agglutination of red blood cells
 w = weak agglutination of red blood cells

the genus *Peromyscus* and perhaps in giving some genetic insight into the question of species formation. The significance of this study, however, is not in solving these questions but rather in providing the background information and means to make the pursuit of these types of studies possible.

In addition, the description of this blood group system as a complex system establishes the deer mouse as an additional species which could be used in studies of the genetic control and biochemical nature of complex blood group systems.

MATERIALS AND METHODS

Animals

Wild trapped *Peromyscus maniculatus borealis* from the vicinity of Edmonton, Alberta and wild trapped *P. m. osgoodi* from Montana were used for isoimmunization and breeding programs. Blood samples for typing purposes were obtained from wild mice from the North West Territories, British Columbia, Montana, Colorado and Michigan, as well as from the laboratory stocks at the University of Michigan and Michigan State University.

Matings and Births in Captivity

Approximately 600 adult mice were kept paired in captivity and allowed to raise their litters. Pairings were made without regard to blood group phenotype and often involved mice from different trapping locations. Each pair was kept in a plastic cage (11-1/2" x 7" x 5") and the male was not usually removed after the birth of a litter. Light conditions in the animal quarters matched natural light conditions and this brought the mice into breeding condition in spring. During the course of the spring and summer breeding season pairs have produced as many as five litters. Some animals paired in 1967 were still producing litters in 1969. Mice born in captivity were bled and typed when they were approximately 30 days old.

Blood Collection and Treatment

Blood samples were obtained from mice by inserting a Natelson type heparinised blood collecting tube¹ into the suborbital canthal sinus (Rasmussen and Koehn, 1966). About 0.5 ml of blood (less from small animals) could be obtained and fatalities were rare. Animals were anaesthetized

prior to blood collecting by injecting pentobarbital sodium² (50 mgm/kg body weight) dissolved in buffered saline³ intraperitoneally. It was found later that this was not necessary and that enough blood for typing could be collected from unanaesthetized animals.

Blood was collected in 1 ml plastic centrifuge tubes.¹ Within 30 minutes of collection, cells and serum were separated by centrifugation at 5000 x g for 5 minutes. The serum was removed by pipette and, if required, was stored in small plastic Microfuge tubes⁴ in a chest freezer at -40° C. The cells were resuspended in an equal volume of buffered saline and were then ready for typing. Typing reactions were clearer if the cells were allowed to stand overnight at 4° C.

If typing reagents were unavailable the cells were resuspended in an equal volume of a glycerol-citrate mixture, prepared by mixing 4 volumes of glycerol with 6 volumes of 5% trisodium citrate solution. The cells could then be stored in a frozen state at -40° C until required. The frozen cells were reconstituted as follows (Chaplin, H. and Mollison, 1953):

- (1) Three solutions were prepared containing respectively 16%, 8%, and 4% of glycerol (w/v) in 3% trisodium citrate.
- (2) The tube of blood was thawed and two or three drops were transferred to another 1 ml centrifuge tube. This was filled to the top with the 16% glycerol solution, mixed well and centrifuged at 3000 x g for 5 minutes.
- (3) The supernatant was replaced with the 8% glycerol solution, then with the 4% solution, and finally with a 1% saline solution.
- (4) The original tube could be thawed and refrozen more than once.

Immunization Procedure

Blood typing reagents were prepared by isoimmunization of recipient mice with individual donor erythrocytes. Age and sex were not factors in the choice of animals. The initial injection consisted of an emulsion of 0.05 ml of packed washed red blood cells and 0.2 ml of Freund's Adjuvant.⁵ This was injected subcutaneously into the shoulder region of the mouse. Twenty-one days later 0.2 ml of a 50% suspension of erythrocytes in buffered saline was injected intraperitoneally and seven days later the recipient mouse was bled and the unabsorbed serum was tested for hemagglutination with the donor and control cells. Most antisera contained a non-specific antibody which became non-reactive at titers greater than 1:2. Antisera were usually used at titers of 1/16 although this was varied depending on the immune response of the individual deer mouse.

Some of the immunized animals received additional booster injections consisting of approximately 0.2 ml of cells made up to a 50% cell suspension in buffered saline. One group received a booster injection three weeks after being bled (four weeks after their second injection) and were bled again in six days. The results were not satisfactory as the animals did not show an increase in antibody titer, in fact some of the animals showed a drop in antibody titer. A second group received a booster approximately 16 weeks after the second injection. These animals generally did respond with increased titers.

Blood Typing Techniques

Blood was typed at room temperature using the Chown capillary tube technique (Dunsford and Bowley, 1967). Capillary tubes,⁶ 90 mm long with an internal diameter of 0.4 mm, were broken in half and one end was dipped

into the 50% cell suspension just long enough for 1-2 mm of blood to layer behind the antisera. Care was taken to insure that no air bubbles separated the two layers. The tubes were inverted so that the cell layer was on top and the lower end was embedded in plasticine. The tubes were tilted at about a 45° angle from the vertical. The red blood cells fell through the serum and a positive result appeared as an interrupted granular line of agglutinates. In the absence of agglutination a smooth unbroken line resulted (Fig. 1). Some positive results were obvious in 5 minutes but final readings were recorded after 30 minutes.

During the course of this study many sera from unimmunized mice were tested for hemagglutination prior to immunization and were found to be generally free of natural antibodies as reported earlier by Rasmussen (1965). Occasionally some sera (neat) reacted with cells in the capillary tube but the reaction did not resemble a positive agglutination reaction. A clumping occurred in which the clumps seemed fuzzy and the tail of cells in the tube was usually smooth. This reaction disappeared at serum titers greater than 1/1. A similar phenomenon has also been associated with certain cells. These cells reacted non-specifically with non-immune sera and yet reacted specifically with certain immune sera, discernible by a true agglutination reaction. This type of phenomenon was rare and did not interfere unduly with normal typing procedures.

Frozen cells could be reconstituted and typed as fresh cells; reconstitution, however, was often accompanied by hemolysis which interfered with normal agglutination reactions. An alternative method for typing frozen specimens was devised in which potent antisera were diluted with the 16% glycerol trisodium citrate solution. Frozen cells could then be typed upon thawing with no reconstitution necessary. Antiserum which was

Figure 1. Chown capillary tube test showing negative and positive agglutination reactions.

normally used at a titer of 1/64 was used at a titer of 1/16 when diluted in the glycerol solution. Typing reactions were identical between a panel of fresh cells and frozen cells (reconstituted in 16% glycerol) from 65 mice.

Absorption and Elution Techniques

Absorption and elution techniques were used on antisera thought to contain a mixture of antibodies. The absorption technique consisted of simply mixing 2 volumes of appropriate cells with 1 volume of antiserum and allowing the mixture to stand at room temperature for 30 minutes. The mixture was then centrifuged at 3000 X g for 2 minutes and the supernatant serum was removed and tested against another sample of the cells used for the adsorption. If the serum still agglutinated these cells a second adsorption was done.

An elution technique (Wiener, 1957) was used to remove the adsorbed antibody. The procedure for this technique was as follows:

- (1) The cells were washed three times in saline.
- (2) After the last wash the saline was removed and the cells were frozen at -20° C.
- (3) The cells were then thawed and 10 volumes of 50% (v/v) pre-cooled ethanol (-6° C), was added and mixed.
- (4) This mixture was then refrozen at -20° C for at least 30 minutes, re-thawed, and then centrifuged at 5000 X g for 5 to 10 minutes.
- (5) The supernatant was discarded and the tube was refilled with distilled water, mixed thoroughly, and centrifuged.
- (6) The supernatant was discarded and replaced with isotonic saline, using a volume of saline equivalent to the original volume of packed cells. This was mixed thoroughly and incubated at 37° C for 30 to 60 minutes.
- (7) The mixture was centrifuged and the supernatant removed to a clean

tube. This was the eluate and could then be tested for antibodies.

Strategy for Defining a Blood Group System

A study which involves the characterization of a blood group system must initially be concerned with the detection of red blood cell antigens. Since the detection of a specific antigen is dependent upon its reaction with a specific antiserum, the first step towards defining a blood group system involves the production of antisera either by isoimmunization or by heteroimmunization.

A detailed account of the production of isoimmune antisera for the characterization of the Pm blood group system is given in the first section of Results. However, it may prove useful at this point to outline briefly the general methodology followed in detection and elucidation of a blood group system by specific antisera.

It is apparent that with no means to characterize the red blood cells the first immunization program must essentially consist of a series of random isoimmunizations amongst individuals (e.g., Birdsall's immunizations mentioned in the Introduction). Since the production of specific antisera involves mice in a limited number of phenotypic combinations, it is probable that the antisera produced by random immunization would be very unlikely to completely define a blood group system as all possible specificities of antisera may not be produced. In addition, the antiserum which is produced may contain antibodies directed against more than one antigen (which may or may not belong to the same system).

The results of such a series, however, will allow for a limited characterization of the red cells and may suggest a preliminary hypothesis concerning a blood group system. With this information a second series of

immunizations can be set up. Thus the second series involves animals typed with the reagents from the first series and some predictions can be made as to its outcome. The results of this second series serve to substantiate, modify, or reject the preliminary hypothesis. As each series is completed the predicted outcome of successive immunizations becomes more accurate, due to greater understanding of the blood group system involved and also due to greater familiarity with the typing procedure and typing reagents.

In addition to defining the antigens, a hypothesis about a blood group system must also be concerned with the manner of inheritance of the antigens. This final information comes from the analysis of breeding data which shows the manner of inheritance of the blood group phenotype.

Footnotes

¹Fisher Scientific Co., Montreal, Quebec

²'Nembutal,' Abbott Laboratories Ltd., Montreal, Quebec

³'Bacto' Heamagglutination Buffer, Difco Lab., Detroit, Mich., U.S.A.

⁴Beckman Instruments Inc., Toronto, Ontario

⁵Colorado Serum Co., Denver, Colorado, U.S.A.

⁶Certified Blood Donor Service Inc., Jamaica, N.Y., U.S.A.

RESULTS

Immunization Series Involved in the Production of Typing Reagents

1. Preliminary work. As mentioned in the Introduction, a series of isoimmunizations of deer mice were carried out in the summer of 1967 by D. A. Birdsall. His results and antisera were kept and made available to me at the beginning of my work one year later. Table I shows the results of his final isoimmunization program. This table suggests that most antisera roughly exhibited one of two patterns of reactivity or were non-reactive. Sera which gave reactions characteristic of one pattern were designated as anti-A and sera which gave reactions characteristic of the second pattern were designated as anti-B.

An arbitrary sample of approximately 50 deer mice were then tested with these reagents; of these about 1/3 could be typed as A, 1/3 as B, 1/3 as AB, and three animals did not react with any of the reagents. The variation of the reactivity of the antisera in each class (see Table I), the relative lack of heterozygotes, and the three non-reacting individuals was at that time attributed to low antibody titers, possibly due to deterioration of the sera during storage or to a low titer of antibody in the original specimen. Assuming that this could account for the discrepancies, it was hypothesized that these reagents were detecting a two allele, three phenotype blood group system. An immunization program was then initiated to test this hypothesis.

2. First isoimmunization series. This immunization program was carried out using animals which had been typed as A, B, or AB with the use of Birdsall's reagents. The choice of donor and recipient mice is outlined in Table II which also indicates the antibodies expected.

Table II. Program of the first isoimmunization series

Recipient Mouse	Blood Group	Donor Mouse	Blood Group	Antibody Expected
765	B	754	A	Anti-A
739	B	751	A	Anti-A
776	B	770	A	Anti-A
636	B	758	A	Anti-A
47	B	753	AB	Anti-A
728	A	762	B	Anti-B
751	A	739	B	Anti-B
770	A	776	B	Anti-B
732	A	636	B	Anti-B
42	A	779	B	Anti-B
48	A	743	B	Anti-B
731	AB	733	AB	nil
733	AB	731	AB	nil
734	AB	654	AB	nil
763	AB	729	AB	nil
27	AB	730	AB	nil

Immunizations consisted of the injection of donor erythrocytes into the recipient deer mouse. Blood grouping was based on reactions with Birdsall's reagents and the prediction of antibodies was based on the hypothesis that a two allele blood group system was involved.

Expectations were based on the hypothesis that a two allele blood group system was involved. Then, an A mouse if injected with B cells should produce anti-B, a B mouse if injected with A cells should produce anti-A, and an AB mouse if injected with either A or B cells should not produce any antibodies.

Table III shows the results achieved by this program and indicates the pattern of reactions based on Birdsall's reagents which were thought to characterize anti-A and anti-B antisera. In general the sera produced fell into one of three classes.

Class 1: *Sera which gave results roughly parallel to those of the expected anti-B.* These sera were produced by mice 47, 728, 751, 770, 733, and 48. Of these, however, only four were expected to produce anti-B antisera, one was expected to produce anti-A antiserum, and one was presumed to be an AB heterozygote and was not expected to produce any antibodies.

Class 2: *Sera which did not react with any of the cells.* It was expected that AB heterozygotes would not produce antibodies. However, none of the mice which produced non-reacting sera had been typed as AB; two of these mice had been typed as B and were expected to produce anti-A antisera, and one had been typed as A and was expected to produce anti-B antiserum.

Class 3: *Sera which did not fit into either of the above classes.* These sera did not react uniformly nor did they give results consistent with the proposed anti-A antiserum. Of these antisera four were produced by presumed heterozygotes and two were produced by mice which had been expected to produce anti-A antiserum.

From these results it seemed likely that Birdsall's reagents

Table III. Reactions of the first series of isoimmune antisera to red blood cells of individual deer mice

Deer mice erythrocytes	Isoimmune antisera							Birdsall's reagents						
	47	728	751	770	733	48	763	636	776	731	27	734	Anti-A	Anti-B
47	-	-	-	-	-	w	+	-	-	+	+	-	-	+
728	-	-	-	-	-	-	+	-	+	+	+	-	+	-
751	-	-	-	-	-	-	-	+	+	-	+	+	+	-
770	-	-	-	-	-	-	-	+	+	-	+	-	+	-
733	-	w	-	-	-	-	+	+	+	+	+	w	+	+
48	-	-	-	-	-	-	-	+	-	+	-	+	+	-
42	-	-	-	-	-	-	+	-	+	+	+	+	+	-
732	-	-	-	-	-	-	+	-	+	+	+	+	+	-
765	-	-	-	-	-	w	+	+	-	+	+	+	-	+
739	+	+	+	+	+	+	-	-	-	+	+	+	-	+
776	+	+	+	+	+	+	-	-	-	+	-	+	-	+
636	+	+	+	+	+	+	-	-	-	-	-	w	-	+
731	+	+	+	+	+	+	-	-	+	-	+	-	+	+
734	+	+	+	+	+	+	+	-	+	+	+	-	+	+
763	+	+	+	+	+	+	-	-	+	-	+	+	+	+
27	+	+	+	+	+	+	-	-	-	+	-	+	+	+

Isoimmune antisera produced as outlined in Table II. 42 produced a non-specific antibody and 765, 739, and 732 did not produce any antibodies.

and the proposed theory were inadequate to describe the blood group system.

Further analysis of Table III reveals that there was no single antiserum which would agglutinate the cells of all mice which produced anti-B. This indicated that the mice which produced anti-B antisera did not all have the same blood group phenotype. The simplest blood group system which could accommodate this observation would be a three allele, six phenotype, blood group system. Then, designating the antigens determined by these alleles as A, B, and C, mice which produced anti-B antisera could have one of three phenotypes, A, AC, or C, and these should be detectable with the use of two antisera (anti-A and anti-C).

Examination of Table III for two such antisera revealed that there were several combinations of two antisera which could agglutinate the cells of all the mice which produced anti-B antisera. However, of these combinations only three were thought to warrant consideration because the other combinations reacted with cells which contain the B antigen in such a way that mice could be described as having three antigens (ABC phenotype).

The three combinations of antisera to be considered were 636 and 763, 636 and 731, and 636 and 27. Serum 636 was common to all combinations and was tentatively designated anti-A. Antiserum 763 reacted with relatively fewer specimens than either 731 or 27 and for this reason it was thought that this serum might be more specific and less likely to contain a mixture of antibodies. Thus antiserum 763 was designated anti-C.

A random sample of mice was then tested with these two reagents and an anti-B antiserum (770 or 751). Thirty-four animals were tested and with the use of the three antisera each animal could be assigned a blood group as shown in Table IV. As this table shows there were many more

Table IV. Distribution of blood group phenotypes in an arbitrary sample of mice

Blood Group Phenotypes	A	B	C	AB	AC	BC
Number of Mice	11	10	7	3	1	2

homozygotes than heterozygotes. It was realized at the time that some of these homozygotes may in fact be heterozygotes. A mistyping such as this could arise as the result of using a weak antiserum which could react visibly with an antigen only if its corresponding allele was in the homozygous state (dosage effect). During the typing procedures it had become evident that the antibody titers of the sera dropped rapidly. This was believed due to the thawing and refreezing of the entire specimen several times and thereafter the new antisera were divided into small aliquots prior to freezing.

The fact that each animal could be assigned a blood group and the fact that no animal reacted with all three antisera suggested that these reagents were in fact detecting a three allele, six phenotype, blood group system. A second series of immunizations was then set up to test this hypothesis.

3. Second series of isoimmunizations. The second series of isoimmunizations is outlined in Table V. This table indicates the blood group phenotype of recipient and donor mice and lists the antibody specificities expected based on the phenotypes of the mice involved in specific immunizations.

It should be kept in mind that although all the animals had been typed as homozygotes there was a strong possibility that some were heterozygotes (see above).

Table VI outlines the pattern of reactions of the antisera produced in this immunization series and also indicates the expected pattern of reactions of anti-A, anti-B, and anti-C as determined by the antisera from the previous immunization program. The following analysis is based on the expectations outlined in Table V.

Table V. Second program of isoimmunizations

Recipient Mouse	Blood Group	Donor Mouse	Blood Group	Antibody Expected
16	B	374	A	Anti-A
294	B	677	A	Anti-A
164	B	296	A	Anti-A
313	C	679	A	Anti-A
469	B	539	A	Anti-A
101	C	92	B	Anti-B
679	A	294	B	Anti-B
374	A	16	B	Anti-B
296	A	164	B	Anti-B
622	C	96	B	Anti-B
96	B	622	C	Anti-C
92	B	101	C	Anti-C
677	A	313	C	Anti-C

Predictions of antibodies are based on the hypothesis that a three allele blood group system is involved.

Table VI. Reactions of the second series of isoimmune antisera to red blood cells of individual deer mice

Deer mice erythrocytes	Isoimmune antisera										Anti- A*			Anti- B*			Anti- C*		
	16	294	164	313	469	101	679	374	296	622	96	92	677						
294	+	-	-	-	-	+	+	+	+	+	-	-	-	-	+	-	-	-	-
96	+	-	-	-	-	+	+	+	+	+	-	-	-	-	+	-	-	-	-
92	+	-	-	-	+	+	+	+	+	+	-	-	-	-	+	-	-	-	-
16	-	-	-	-	+	+	+	+	+	+	-	-	-	-	+	-	-	-	-
164	+	-	-	-	-	+	+	+	+	+	-	-	-	-	+	-	-	-	-
469	+	-	-	-	-	+	+	+	+	+	-	-	-	-	+	-	-	-	-
622	-	-	-	-	-	-	+	+	+	-	+	+	+	-	-	-	+	+	+
101	+	-	-	-	-	-	+	+	+	+	+	+	+	-	-	-	-	-	-
313	+	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-
374	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-
296	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-
677	+	-	-	-	+	-	-	-	-	+	-	-	-	+	-	-	-	-	-

Isoimmune antisera produced as outlined in Table V. *Antisera from the first series of isoimmunization.

Anti-A. Five non-A animals (16, 294, 164, 313, 469) were expected to produce anti-A antisera when injected with A positive cells. However, only two of these mice (16 and 469) produced any antibodies and only mouse 16 apparently produced the predicted anti-A. This observation was in keeping with the possibility that the previous anti-A antiserum was weak and only detected A homozygotes. Then it would be conceivable that the four other mice in this group did not produce anti-A antisera because they themselves carried the A antigen as well as one other. Consistent with this suggestion was the fact that serum 16 which reacted with all the proposed A positive cells also reacted with the cells of these four animals. Typing with this antiserum led to a reclassification of three other mice (101, 96, 92) and these are noted in Table VII. The other mouse in this group (469) which produced an antibody produced a very weak antibody with characteristics unlike anti-A, anti-B or anti-C and has not been further analysed.

Anti-B. Five animals (101, 679, 374, 296, 622) were expected to produce anti-B. Four of these mice (101, 679, 374, 296) produced antisera which gave results similar to the expected anti-B. One of these antisera (101) reacted exactly as predicted (it agglutinated cells of mice 294, 96, 92, 16, 164 and 469). The other three mice produced antisera which reacted as the anti-B with one important difference. These sera also reacted with the cells of mice 101 (which was non-B, see above) and 622 (a C homozygote). Investigation of these antisera revealed that 101 cells could be used to adsorb an antibody component leaving antisera with the characteristics of the predicted anti-B. The antibody which adsorbed to 101 cells was designated anti-X. A more complete account of this antibody is found on pages 40-45.

Table VII. Summarized results of the second program of isoimmunizations

Recipient Mouse	Revised Blood Group	Donor Mouse	Revised Blood Group	Antibody Expected	Antibody Produced
16	B	374	A	Anti-A	Anti-A
294	AB	677	A	Nil	Nil
164	AB	296	A	Nil	Nil
313	AC	679	A	Nil	Nil
469	AB	539	A	Nil	Unknown
101	AC	92	AB	Anti-B	Anti-B
679	A	294	AB	Anti-B	Anti-B & Anti-X
374	A	16	B	Anti-B	Anti-B & Anti-X
296	A	164	AB	Anti-B	Anti-B & Anti-X
622	C	96	AB	Anti-A & Anti-B	Anti-A & Anti-B
96	AB	622	C	Anti-C	Anti-C
92	AB	101	AC	Anti-C	Anti-C
677	A	313	AC	Anti-C	Anti-C
*279	A	63	AC	Anti-C	Anti-C
63	AC	279	A	Nil	Nil
472	B	469	AB	Anti-A	Anti-A

*The phenotype of these three animals was unknown prior to their use and therefore were not included in Table VI. Revised blood group based on reactions of antisera produced in this series.

The remaining mouse (622), expected to produce anti-B, produced an antibody which reacted with all tested cells except its own. It should be noted here that 622 was unique in that it was the only C homozygote used in this series (see Table VII). Analysis of this situation showed that with the use of serum 16 as anti-A the donor mouse (96) was retyped as an AB mouse. Consequently 622 could then be expected to produce anti-A and anti-B. Subsequent absorption studies showed this to be the case.

Anti-C. Three animals (96, 92, 677) were expected to produce anti-C and as can be seen in Table VI and as evidenced by their positive reactions to cells of mice 622, 101 and 313, they did.

Concurrent with this program three immunizations were carried out which involved animals of unknown phenotype. These animals were subsequently typed with the reagents produced in this series and these results are included in Table VII. This table also indicates the antibodies expected and the antibodies produced by these mice. The anti-A produced by one of the mice of this group reacted in a manner consistent with serum 16 and this added further support to the assumption that serum 16 was reacting only with the A antigen.

Table VII summarizes the results of this series including the re-typing of the mice involved. The results substantiate the hypothesis that a three allele, six phenotype, blood group system was being detected. The reagents produced by this series were then used to type a large sample of mice which included wild trapped mice and breeding stock. These results, in showing that all mice reacted to one or two antisera, added further support to the above hypothesis. None of the typing results indicated that the X antigen was an alternative antigen to A, B, or C. However, this did not necessarily rule out the possibility that X specificity was

determined by the locus controlling the A, B, and C specificity.

4. Third and fourth isoimmunization series. Two additional immunization programs were carried out to increase the supply of antisera. Table VIII describes the third immunization program and indicates the antibodies expected based on the three allele hypothesis. The anti-X phenomenon which was associated with three of the five anti-B antisera in the last series was not understood at this point and consequently the only anti-X predicted for this series was the antiserum produced by the specific immunization of an AC mouse (318) with CX cells (133).

Table IX shows the results of this series and indicates that the results are generally as expected. However, further testing of the antisera revealed two points of special interest.

(1) The anti-X (318), which reacted strongly with X positive cells, was additionally found to react with all B positive cells (the reaction was somewhat weaker).

(2) The anti-B antisera of this series were found to share the same characteristics of the three anti-B antisera of the second series in that as well as reacting with all B positive cells these sera reacted with non-B, X positive cells.

These results imply that X positive cells and B positive cells share a common antigenic property. Additionally, the association of X and B suggests that the X antigen is in fact part of the same blood group system which involves A, B, and C.

The fourth immunization program is outlined in Table X. It was thought that the problem of producing a cross-reacting anti-B (or anti-BX) antiserum could be avoided in this series by injecting B cells into non-B, X positive recipients, that is mice of blood type ACX, CX, or AX (no

Table VIII. Third program of isoimmunization

Recipient Mouse	Blood Group	Donor Mouse	Blood Group	Antibody Expected
100	BC	389	AB	Anti-A
317	BC	678	AB	Anti-A
95	B	135	A	Anti-A
163	B	373	A	Anti-A
166	B	316	A	Anti-A
373	A	163	B	Anti-B
313	AC	166	B	Anti-B
678	AB	317	BC	Anti-C
278	A	318	AC	Anti-C
389	AB	100	BC	Anti-C
318	AC	133	CX	Anti-X

Predications of antibodies are based on the hypothesis that a three allele blood group system is involved.

Table IX. Reactions of the third series of isoimmune antisera to red blood cells from individual deer mice of known blood group phenotype

Cells	Group	Isoimmune antisera							
		100	317	95	163	166	373	313	389 318
95	B	-	-	+	-	-	+	+	- +
163	B	-	-	+	-	-	+	+	- +
166	B	-	-	+	-	-	+	+	- +
100	BC	-	-	+	-	-	+	+	+ +
317	BC	-	-	+	-	w	+	+	+ +
678	AB	+	+	+	+	+	+	+	- +
389	AB	+	+	+	+	+	+	+	- +
373	A	+	+	+	+	+	-	w	- -
278	A	+	+	+	+	+	-	-	- -
313	AC	+	+	+	+	+	-	-	+ -
318	AC	+	+	+	+	+	-	-	+ -

Antibody Specificity	Anti-A	Anti-A	Non-Specific	Anti-A	Anti-A	Anti-B(X)*	Anti-B(X)*	Anti-C	Anti-X

Isoimmune antisera produced as outlined in Table VIII. Sera 678 and 278 are not shown in this table. Both sera gave results commensurate with anti-C but also reacted with a number of other individuals when tested at titers of 1:1, testing at a later date revealed that the antibody titer had dropped to the point where the specificities of these antisera could not be ascertained.

* see text (page 29).

Table X. Expected and observed results of the fourth series of
isoimmunizations

Recipient Mouse	Blood Group	Donor Mouse	Blood Group	Antibody Expected	Antibody Produced
1223	B	1394	A	Anti-A	Anti-A
1390	B	1385	A	Anti-A	Anti-A
1118	ACX	1384	B	Anti-B	Anti-B
1033	ACX	1340	B	Anti-B	Anti-B
1216	ACX	1383	B	Anti-B	Anti-B
1226	ACX	1316	B	Anti-B	Anti-B
1230	A	1408	AC	Anti-C	Anti-C
1130	AB	1407	BC	Anti-C	Anti-C
1116	AB	1411	BC	Anti-C	Anti-C
1389	AB	1363	BC	Anti-C	Anti-C (variant)
1028	AB	700-46	CX	Anti-C	Anti-C
1119	AB	1339	BC	Anti-C	Anti-C
1227	A	700-41	CX	Anti-C	Anti-CX
1129	A	700-44	CX	Anti-C	Anti-CX

Seven mice in this series are not included in this table. They produced very weak antibodies whose specificities were masked by non-specific agglutination at serum titers of 1:1 (see Materials and Methods). Subsequent dilutions resulted in very weak antisera which could not be accurately classified.

mice of AX phenotype had been detected at this point). Table XI gives the results of this series and shows that this method of immunization did produce the desired anti-B antisera. It should be pointed out that although the problem of producing anti-BX antisera was corrected in this series the procedure was not extended to all immunizations and consequently two unwanted anti-CX antisera were produced. Unlike B positive cells, however, not all C positive cells carry the X antigen.

The antisera produced by the third and fourth series were used to type a large number of animals and these results suggest strongly that the proposed three allele hypothesis is correct. Moreover the two instances of non-random association of X with this blood group system (all B positive individuals are X positive and no individuals were found to be AX) indicates that there is close linkage of X to this blood group system and raises the possibility that the system may be more complex in nature.

Inheritance of Antigens A, B, and C

1. Genetic control of antigens A, B, and C.

Over 3500 mice, which included wild trapped animals and breeding stock, were tested with the three types of antisera. The three reagents anti-A, anti-B, and anti-C detected six phenotypes, A, B, C, AB, AC, and BC. Cells from all individual mice reacted with one or two reagents but no individual reacted with all three. These serological observations were consistent with the proposed hypothesis that these reagents were detecting a blood group system which was under the genetic control of three co-dominant alleles of one locus.

The breeding data shown in Table XII substantiates this hypothesis. This table shows that the phenotypes and their ratios produced among the

Table XI. Reactions of the fourth series of isoimmune antisera with red blood cells from individual deer mice of known blood group phenotypes

Cells	Blood Group	Isoimmune antisera*													
		1223	1390	1118	1033	1216	1226	1230	1130	1116	1389	1028	1119	1227	1129
1129	A	+	+	-	-	-	-	-	-	-	-	-	-	-	-
1230	A	+	+	-	-	-	-	-	-	-	-	-	-	-	-
1227	A	+	+	-	-	-	-	-	-	-	-	-	-	-	-
1130	AB	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1116	AB	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1119	AB	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1028	AB	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1389	AB	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1220	B	-	-	+	+	+	+	+	+	+	+	+	+	+	+
1390	B	-	-	+	+	+	+	+	+	+	+	+	+	+	+
1077	B	-	-	+	+	+	+	+	+	+	+	+	+	+	+
1223	B	-	-	+	+	+	+	+	+	+	+	+	+	+	+
1219	B	-	-	+	+	+	+	+	+	+	+	+	+	+	+
1033	AC	+	+	-	-	-	-	-	+	+	+	+	w	+	+
1226	AC	+	+	-	-	-	-	-	+	+	+	+	w	+	+
1216	AC	+	+	-	-	(+)	+	+	+	+	+	+	w	+	+
1127	AC	+	+	-	-	-	+	+	+	+	+	+	+	+	+
1388	AC	+	+	-	-	-	+	+	+	+	+	+	+	+	+
1232	AC	+	+	-	-	-	+	+	+	+	+	+	+	+	+
1118	AC	+	+	-	-	-	+	+	+	+	+	+	+	+	+
1079	AC	+	+	-	-	-	+	+	+	+	+	+	+	+	+
Antibody		Anti-A	Anti-A	Anti-B	Anti-B	Anti-B	Anti-B	Anti-C	Anti-C	Anti-C	Anti-C*	Anti-C	Anti-C	Anti-CX	Anti-CX

Isoimmune antisera produced as outlined in Table X. *variant

Antiserum 1216 was tested at a titer of 1/4 and was not further investigated. It was never used as a typing reagent.

Table XII. Breeding analyses of blood group phenotypes in *Peromyscus maniculatus*

Phenotype of Mating ♀ ♂	Number of Pairs	Number of Litters	Total Offspring	Mean Litter Size	Number of Offspring of each Phenotype				Expected Ratio	Degrees of Freedom	Chi-Square Analyses	
					A	AB	B	BC	AC	C	χ ²	P
A X AB	20	40	198	5.0	98	100	-	-	-	-	1:1	0.02 >.8
AB X A	11	30	139	4.6	81	58	-	-	-	-	1:1	3.81 >.05
A X BC	3	8	43	5.4	-	22	-	-	21	-	1:1	0.02 >.8
BC X A	3	8	41	5.1	-	20	-	-	21	-	1:1	0.04 >.8
A X AC	5	8	42	5.2	18	-	-	-	24	-	1:1	0.75 >.3
AC X A	7	13	59	4.5	26	-	-	-	33	-	1:1	0.92 >.3
B X AB	5	13	71	5.5	-	39	32	-	-	-	1:1	0.69 >.3
AB X B	5	9	38	4.2	-	24	14	-	-	-	1:1	2.63 >.1
B X BC	1	2	10	5.0	-	-	8	2	-	-	1:1	3.6 >.05
B X AC	3	6	30	5.0	-	15	-	15	-	-	1:1	0.0 >.9
AC X B	1	2	9	4.5	-	4	-	5	-	-	1:1	0.11 >.7
C X AB	1	1	4	4.0	-	-	-	2	2	-	1:1	0.0 >.9
AB X C	1	4	28	7.0	-	-	-	14	14	-	1:1	0.0 >.9
C X BC	1	4	14	3.5	-	-	-	6	-	8	1:1	0.3 >.5
C X AC	1	2	8	4.0	-	-	-	-	5	3	1:1	0.5 >.7
AB X AB	13	24	126	5.2	32	67	27	-	-	-	1:2:1	0.9 >.5
AC X AC	5	7	42	6.0	6	-	-	-	26	10	1:2:1	3.1 >.2
AC X AB	9	26	117	4.5	35	26	-	27	29	-	1:1:1:1	0.95 >.8
AB X AC	4	10	48	4.8	12	5	-	11	20	-	1:1:1:1	9.5 >.02
BC X AB	3	9	51	5.7	-	18	8	10	15	-	1:1:1:1	4.92 >.1
AC X BC	2	4	20	5.0	-	4	-	5	9	2	1:1:1:1	5.2 >.1
A X A	19	34	155	4.6	155	-	-	-	-	-	-	-
B X B	1	2	8	4.0	-	-	8	-	-	-	-	-
A X B*	16	31	139	4.5	-	139	-	-	-	-	-	-
A X C*	4	10	57	5.7	-	-	-	-	57	-	-	-
B X C*	1	1	3	3.0	-	-	-	3	-	-	-	-

*Reciprocal crosses combined

P values are expressed as greater than the nearest lower decile or centile.

offspring from each type of cross is consistent with the phenotypes and ratios expected in crosses which involve a blood group system controlled by an autosomal locus with three co-dominant alleles.

Because the antigens determined by two of these alleles subsequently proved to be the same as the antigens determined by Rasmussen's Pm^A and Pm^B alleles (see p.45), his nomenclature is used throughout this study. The third allele described in this study is designated Pm^C .

2. Occurrence of segregation distortion. On close examination of the breeding data in Table XII it can be noted that the distributions of phenotypes in the offspring of crosses involving AB females usually have lower p values than their reciprocal crosses. The cross with C is an exception, but this cross involves only one breeding pair with four litters. The cumulative results of crosses involving AB females indicate that these mice transmit a significantly greater number of A than B alleles to their offspring. Table XIII shows the comparative analysis of the transmission of blood group phenotype by heterozygotes. Only those segregations are displayed in which it is possible to distinguish in the phenotypes of the offspring the allele that was inherited from the heterozygous parent in question.

This phenomenon is illustrated in Figure 2, which shows the segregation of alleles in the total offspring from each heterozygous parent. As can be seen in these graphs nearly every AB female transmitted more A alleles than B alleles to their offspring.

3. Discussion. Various explanations can be put forth to account for the observation of a segregation distortion and these are listed below.

Included with the explanations, however, are reasons why each seems unlikely.

(1) *Mistyping of offspring with non-specific antisera.* This seems unlikely

Table XIII. Segregation ratios among tested heterozygotes

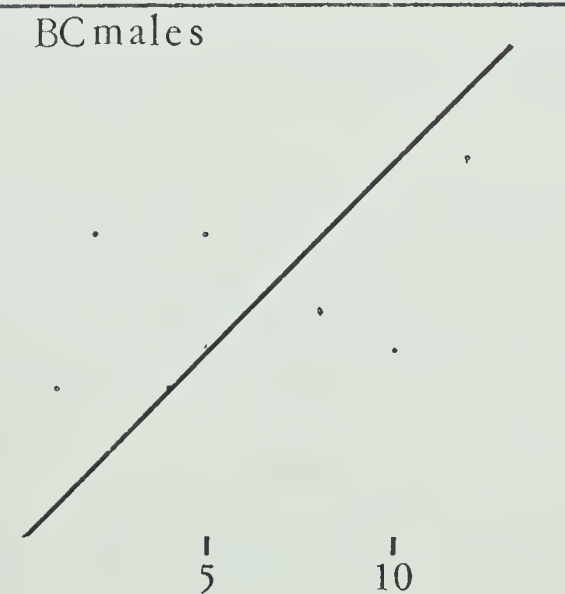
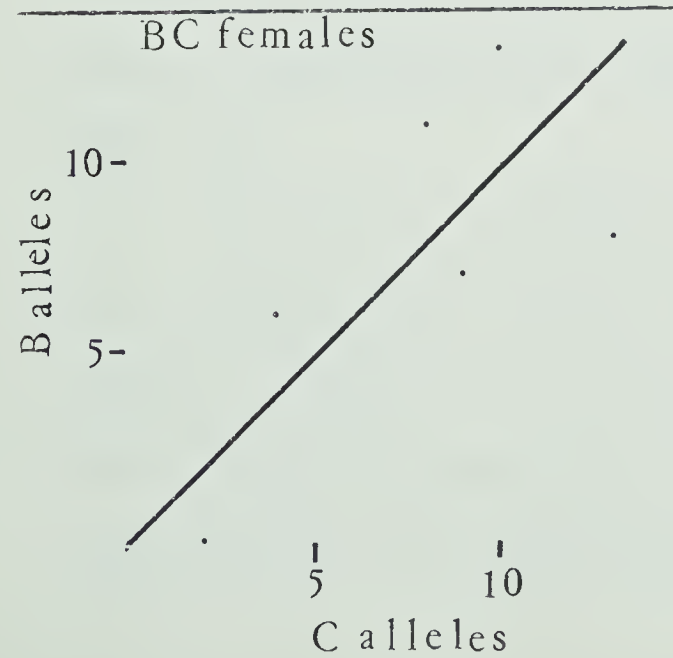
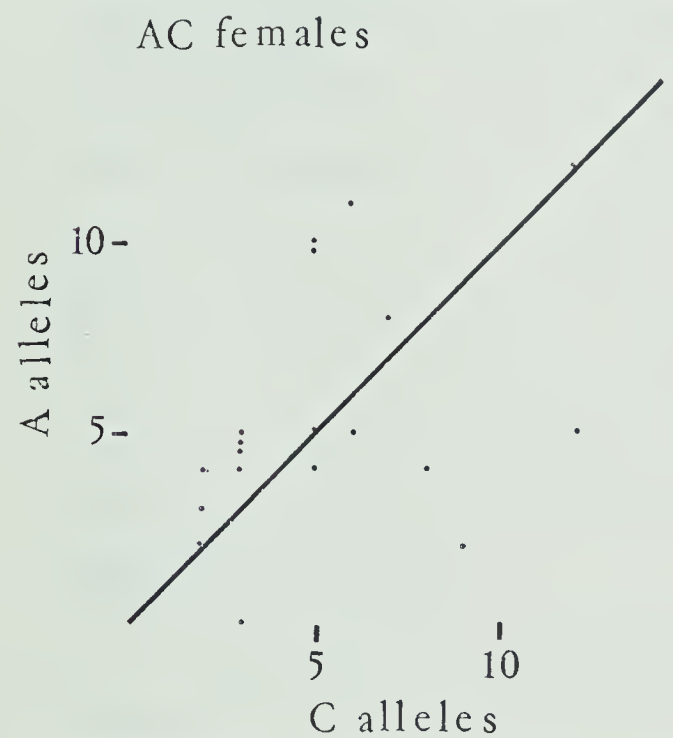
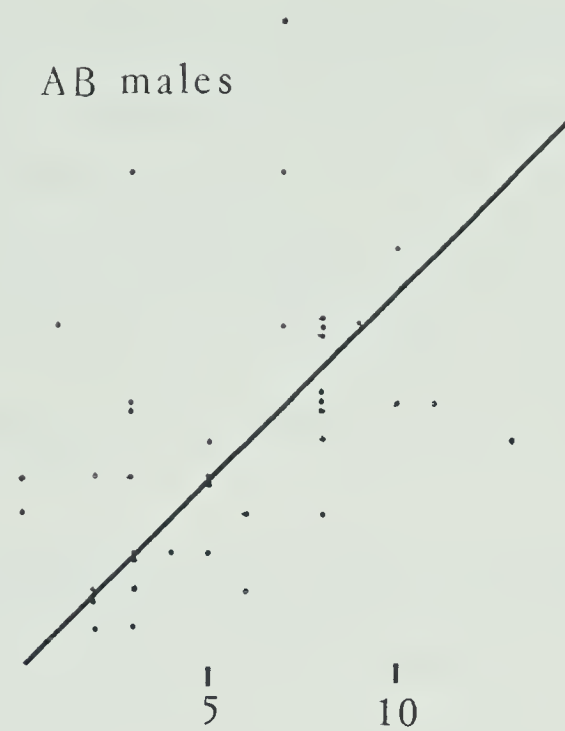
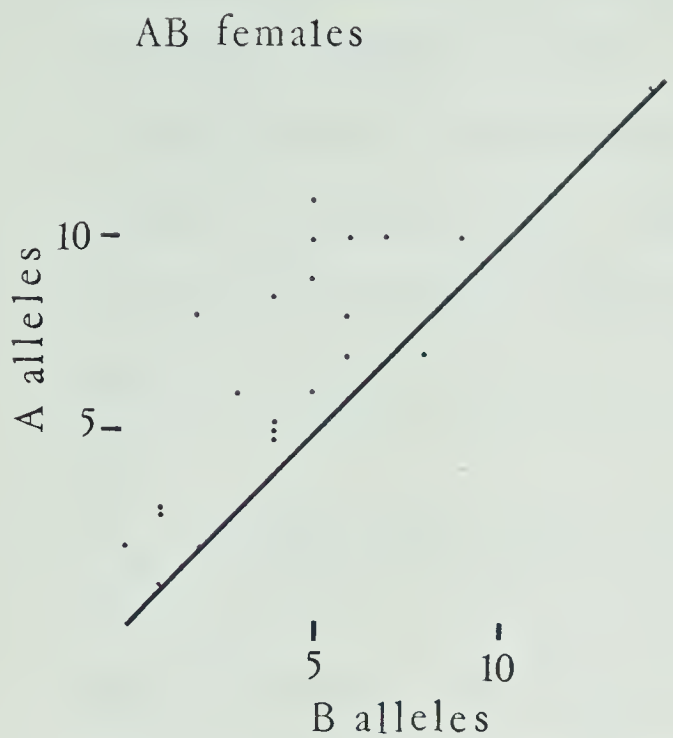
Phenotype of Mate	Test Phenotypes											
	Female				Male				Female			
	AB				BC				AC			
	A	B	A	B	B	C	B	C	A	C	A	C
A	81	58	98	100	20	21	22	21	26	33	18	24
B	24	14	39	32			8	2	4	5	15	15
C	14	14	2	2			6	8			5	3
AB					26	25			61	56	17	31
AC	32	16	64	53			9	11				
BC			33	18					13	7		
Total	151	102	236	205	46	46	45	42	104	101	55	73
χ^2	9.4		2.18		0.0		0.05		0.02		2.52	
P	<.003		>.1		>.9		>.8		>.8		>.1	

Figure 2. Graphs of the segregation ratios of Pm alleles
from each heterozygous parent among its total
number of offspring. The plotted line represents
the expected segregation ratio of heterozygotes.



-ve

+ve



because the distortion is significant only in litters from AB females and not in the reciprocal crosses. No animal in over 3500 tested was found to react with all three antisera which would be expected if one of the reagents was non-specific. Finally, if one of the reagents was detecting more than one antigen it would appear likely that a heterozygous phenotype would be the major factor in the distortion. This, however, was not the case.

(2) *Selective death prior to bleeding dependent upon a certain phenotype.*

This does not seem likely because different phenotypes are deficient in different crosses. For example, in the cross AB X A the AB phenotype appears selected against yet in the cross AB X B, the B phenotype appears selected against. If selective deaths occurred before bleeding, when litter size was determined, it would be expected that the mean litter size in these crosses would be reduced. This is not evidenced in Table XII.

(3) *Heterozygous advantage.* This can be ruled out because in many of the crosses it is the heterozygote which is deficient. The fact that the distortion does not also occur in the reciprocal crosses would also make this explanation unlikely.

(4) *Maternal-fetal incompatibility.* It is difficult to explain the distortion in terms of maternal-fetal incompatibility since the distortion occurs in compatible matings (AB X A) as well as in incompatible matings (AB X AC).

None of these explanations can completely account for this phenomenon. It is possible that it may be a combination of factors; however, any explanation must account for the fact that the distortion is dependent upon the phenotype of the mother alone and that there is no evidence that the mean litter size of these females is decreased. This suggests that some form of

selection may be occurring before implantation, or it may be that for some reason eggs carrying the A allele are more easily fertilized and/or more successful at implantation than eggs carrying the B alleles in an AB female.

Further studies into this phenomenon would seem warranted in an effort to understand this distortion.

Characterization and Genetics of the X Antigen

1. Serological results. During the immunization series a fourth antigen was discovered which did not serve as an alternate antigen to the A, B, or C antigens of the Pm blood group system, yet appeared closely associated with this system. This antigen was tentatively designated anti-X and the observations of its association with the Pm blood group are as follows:

(1) The X antigen was associated with all B positive mice, certain AC and C mice and never with A mice from the populations trapped in the Edmonton area.

(2) Anti-BX was invariably produced by the injection of B cells into non-X positive individuals and conversely anti-B was produced by the injection of B cells into X positive individuals.

(3) Anti-X (produced by the injection of CX cells into an AC recipient) cross-reacted with all B positive cells. The reaction with B positive cells was weaker than with ACX or CX cells.

These observations suggested that B cells had at least two antigenic components, B and X. At this point it was not possible to tell if the X component of B positive cells was identical to the X antigen of ACX and CX cells. The fact that B cells did not react as strongly as CX and ACX cells with anti-X suggested it was not.

Three types of antisera involving X were produced during the immunization series and these included anti-BX, anti-X and anti-CX. Analysis of

these sera with absorption and elution techniques helped clarify the nature of the X antigen and its association with the Pm blood group system. The results of these studies are presented in Table XIV and the analysis is as follows:

Anti-BX. Adsorption of this serum with non-B, X positive cells showed that this serum contained anti-B and an additional antibody which could be eluted from the adsorbing cells. Tests on this eluate revealed that it contained an antibody which reacted with an antigenic component common to all B positive cells and X positive cells, and this antibody was tentatively designated anti-X'.

Anti-X. Absorption analysis revealed that this serum contained a population of antibodies directed against at least two antigenic components. An antibody which adsorbed to the B cells was assumed to be analogous to anti-X' and an antibody which remained in the serum and which reacted specifically with ACX and CX cells was designated anti-X''.

Anti-CX. Based on the foregoing results it was assumed that this serum would contain three antibodies, anti-C, anti-X' and anti-X'', and the results shown in Table XIV confirm this.

2. Inheritance of the X antigen. Preliminary breeding data shown in Table XV indicates that in crosses involving ACX individuals the character X segregates with C. The two individuals which could represent recombinants between C and X were not available for retyping. At the time of testing, their X specificity was simply recorded and never rechecked. If they were true recombinants then the frequency of recombination was 1.3%. One would then expect to see some AX individuals in natural populations in which ACX individuals were present. In testing over 1500 wild-trapped mice from the Edmonton area, no AX individuals were found. It is possible that

TABLE XIV. Absorption analysis of isoimmune antisera involving anti-X

Test Cells	Anti-BX Unabsorbed	Absorbed with ACX Cells	Eluate of the ACX Cells	Anti-X Unabsorbed	Absorbed with B Cells	Anti-CX Unabsorbed	Absorbed with AC Cells	Absorbed * with B Cells
ACX	+	-	+	+	+	+	+	+
B	+	+	+	+	-	+	+	-
AC	-	-	-	-	-	+	-	-
Antisera	Anti-B	Anti-B	Anti-X'	Anti-X'	Anti-X''	Anti-C	Anti-X'	Anti-X''
Specificities	Anti-X'			Anti-X''		Anti-X'	Anti-X''	
						Anti-X''		

* Anti-CX absorbed sequentially first with AC cells and then with B cells.

Table XV. Segregation results of crosses involving mice carrying the X antigen

Cross	Prs.	Litters	Phenotypes expected if X segregates with C (i.e. parental types)				Phenotypes not expected if X segregates with C (i.e. recombinants)	
A x ACX	3	6	A	ACX			AX	AC
		obs.	14	14			0	0
AC x ACX	3	5	A	ACX	CX	AC	AX*	C
		obs.	5	5	6	13	(1)	0
AB x ACX	4	8	A	ACX	AB	BC	AX	AC
		obs.	11	13	11	6	0	0
A x BCX	1	2	AB	ACX			AC	
		obs.	5	5			0	
AC x BCX	1	2	AB	ACX	CX	CB	AC	C
		obs.	2	3	2	3	0	0
AB x BCX	1	2	AB	ACX	B	CB	AC	
		obs.	2	3	1	4	0	
C x BCX	1	3	CX	CB			C*	
		obs.	6	3			(1)	

*The two individuals whose phenotypes were not expected if X segregates with C and were not double-checked at the time of testing.

the "recombinants" were inaccurately described or that AX is lethal in the wild. However one animal captured in the southeast part of Alberta, 400 miles from the Edmonton area, was AX and produced two AX offspring in a litter of six. AX mice were also detected in the Michigan colonies. Incorrect blood typing is the most likely explanation of the assortment between C and X.

The close association of X with the Pm blood group system, as evidenced by the serological results and breeding data, establishes the Pm blood group system as a complex system. Although the data is consistent with the notion that possibly three closely linked loci may be involved: one locus determining A, B, and C specificity, one determining X' or the absence of X' specificity, and one determining X'' or the absence of X'' specificity, it is probably more convenient to describe the blood group system as being controlled by a single complex gene locus. Then without prejudice to the actual genetic mechanism of antigenic determination, it is possible to tentatively describe the Pm locus as if it has at least four alleles, Pm^A , Pm^B , Pm^C , and Pm^{CX} , of which two (Pm^B and Pm^{CX}) can be shown to determine more than one antigenic specificity. Two additional alleles, Pm^{AX} and Pm^X , have been further hypothesized and these are discussed on page 46.

3. Revised nomenclature. It seems very likely that further investigation into this blood group system will reveal even more alleles which would render the above terminology unwieldy in the future. It may prove convenient to continue designating the alleles alphabetically, such that Pm^A , Pm^B , Pm^C , remain as they are and Pm^{CX} becomes Pm^D . Antigenic determinants which may become very numerous can be numbered numerically in order of discovery such that antigenic determinants A, B, C, X', and X'' would be

designated 1, 2, 3, 4, and 5 respectively.

Michigan Colonies

1. Confirmation that the blood group system outlined in this study is determined by the Pm locus. The results outlined in this study were obtained independently of Rasmussen. Rasmussen (1961) originally discovered the Pm blood group system using animals from the laboratory stocks maintained by Dr. Elizabeth Barto, Department of Zoology at the University of Michigan. Blood samples from this colony and a sample of Rasmussen's anti-B antiserum were used to confirm that his Pm locus was involved in this blood group system as well.

Rasmussen's anti-B antiserum was tested against a panel of test erythrocytes and was found to give results which paralleled anti-BX antiserum. His serum was subsequently adsorbed with non-B, X positive cells to leave an antiserum which gave results characteristic of the anti-B antiserum produced in this study.

In addition, descendants of the animals (*P. m. gracilus* and *P. m. bairdi*) used in Rasmussen's original work were tested with the reagents produced by this study and were found to react only with anti-A, anti-B, and anti-X antisera. No C positive animals were detected. These reactions were consistent with the fact that Rasmussen only detected two antigens, A and B. It should be mentioned here that although the C antigen was absent from the above mice, it was detected in the Michigan colonies in animals belonging to the subspecies *P. m. artemisiae*, *P. m. rubidus*, *P. m. rufinus*, *P. m. sonoriensis* and *P. m. labecula*, none of which are native to the Michigan area.

Eighteen *P. polionotus* were also tested and these all reacted only

with anti-A antiserum. Rasmussen had also found that *P. polionotus* reacted only with one of his antisera, anti-A.

2. Possible detection of two additional alleles. Many animals were typed in Michigan with anti-A, anti-B, anti-C and anti-X antisera. The typing reactions of blood samples from certain mice suggested the existence of two additional alleles, Pm^X and Pm^{AX} .

The presence of the Pm^X allele was suggested by the reactions of mice of the subspecies *P. m. labecula*. Nine animals from the J. A. King Laboratory at Michigan State University were tested; two were found to be CX and the other seven were found to be X only. This result need not necessarily be interpreted to mean that X is strictly allelic to A, B, and C; these mice were caught in south central Mexico and it is possible that they have some other antigenic component analogous to A, B, and C which remained undetected with our antisera because it is absent from the donor mice from Alberta.

The possibility of Pm^{AX} allele is suggested by the finding of AX individuals, one from southeast Alberta (*P. m. osgoodi*), and six from a sample of sixteen wild-trapped mice from the Dunbar area of Michigan (*P. m. gracilus*), which were sampled from the laboratory of E. Barto at the University of Michigan. However, these animals may be heterozygous AX individuals (Pm^A/Pm^X). This would eliminate the proposed Pm^{AX} allele but further substantiate the presence of Pm^X allele.

The proof of these alleles, however, will have to depend on further serological tests and breeding data which at this point is unavailable.

3. Other *Peromyscus* species. Mice representing other species of *Peromyscus* were also tested with the four antisera and all gave negative results with the exception of *P. polionotus*, which is a member of the

"*maniculatus* group." The animals which did not react with the antisera included representatives of *P. leucopus*, *P. truei*, *P. crinitus*, *P. interparietalis*, *P. stephani*, *P. eremicus*, *P. difficilis*, *P. mexicanus*, *P. melanophorus*, *P. oaxacensis*, *P. californicus*, and *P. boylei*.

Occurrence and Distribution of the Antigens of the Pm Blood Group System in Natural Populations

1. Hardy-Weinberg equilibrium. Blood group antigens have proved useful as genetic markers in population studies (see Introduction). Population genetics is essentially the study of "the frequencies of different alleles in a population, the interaction of alleles at the same or different loci, and the degree to which such interactions govern gene frequencies themselves" (Wallace, 1968). The role of the population geneticist is not in simply cataloguing gene frequencies but in analysing the data in order to form some generalizations which can predict what the genetic composition of a given population is like at a given moment. Hardy-Weinberg equilibrium has been used extensively as a basis for analysing the data obtained by sampling populations.

The Hardy-Weinberg Law states that in a large random-mating population, in the absence of migration, mutation and selection, both allele frequencies and genotype frequencies are constant from generation to generation and that the genotype frequencies are determined by the allele frequencies (Falconer, 1960). Such a population is said to be in Hardy-Weinberg equilibrium. The distribution of genotypes in such a population can be calculated when the allele frequencies are known. Thus if alleles A and a occur in male and female gametes with frequencies p and q , then the frequencies of AA, Aa and aa which arise through random mating will be p^2 , $2pq$, and q^2 .

Expected zygotic frequencies can therefore be calculated and compared to observed zygotic frequencies. Since Hardy-Weinberg equilibrium is based on a number of assumptions, e.g., random mating, absence of selection, a significant difference between observed and expected zygotic frequencies would indicate that one or more of these assumptions had not been met and would suggest further analysis of the population.

During the years 1968 to 1971 many diverse populations of deer mice from Montana, Northwest Territories, Alberta and British Columbia were sampled by D. A. Birdsall, Dr. D. G. Cameron, and Dr. R. P. Canham. Blood samples from these populations were typed with the three Pm blood group reagents, anti-A, anti-B, anti-C antisera, and although the results of these studies are not complete some of the data is presented here.

The distributions of blood group phenotypes in several populations from the Edmonton area were compared with the expected Hardy-Weinberg distribution. Each allele frequency in a population was calculated as equal to the sum of the frequency of the homozygous class plus one half of every heterozygous class carrying that allele (Wallace, 1968). Thus the frequency of Pm^A was equal to the sum of the frequencies of A mice plus one half the frequency of AB mice plus one half the frequency of AC mice in any given population. Designating the frequencies of Pm^A , Pm^B , and Pm^C as p , q , and r respectively, then $p + q + r = 1$ and the Hardy-Weinberg distribution of genotypes (phenotypes) would be the binomial expansion of this equation. Thus the expected frequencies of A, B, C, AB, AC, and BC were calculated as p^2 , q^2 , r^2 , $2pq$, $2pr$, and $2qr$ respectively. The observed and expected frequencies were compared with the Chi-square test.

In general the observed distribution of blood group phenotypes in each population appeared to be in agreement with Hardy-Weinberg equilibrium.

Table XVI presents the analysis of four such populations from the Edmonton area and shows that none of the observed distributions of phenotypes differ significantly from the expected. This does not necessarily mean that all the assumptions dealing with the Hardy-Weinberg Law were true for each population. As was pointed out earlier, AB females appear to transmit more A alleles than B alleles to their offspring and if this phenomenon was occurring in these populations at least one assumption upon which Hardy-Weinberg equilibrium is based would be violated. However, the effect of the segregation distortion could possibly remain undetected because of the insensitivity of the test or because the effect it created was balanced by another process (e.g., selection). Therefore, although a deviation from Hardy-Weinberg equilibrium suggests that one of the assumptions about the population is invalid it is not true that the lack of a significant deviation from Hardy-Weinberg equilibrium suggests the converse.

2. Variations between deer mice populations. The frequencies of the three Pm alleles, Pm^A, Pm^B, and Pm^C, in various populations are presented in Table XVII. Several populations are divided into two groups, adults and juveniles, and from these it can be seen that the allele frequencies among the parents and offspring are fairly consistent. This agrees with Rasmussen (1964) who tested natural populations in Michigan with anti-A and anti-B antisera and found the observed frequencies of the Pm^A and Pm^B alleles to be uniform between adults and juveniles.

Although the allele frequencies are consistent from generation to generation, they are not consistent from area to area. Marked variation in allele frequency can be seen between populations separated by great distance, e.g., Montana and Northwest Territories, and also between populations relatively close together, e.g., wood lots, Nisku 1 and Edmonton 1.

Table XVI. Observed and expected distribution of blood group phenotypes in four populations of deer mice

Deer Mouse Population	Observed or Expected	Number Tested	Distribution of phenotypes							Degrees of Freedom	Chi-Square Analysis	
			A	AB	B	BC	AC	C	χ^2		P	
Edmonton 1 Adults	Obs.	138	67	45	14	4	8	0				
	Exp.		63.4	52.2	10.7	3.3	8.0	0.3	4	2.73	>.5	
Edmonton 1 Juveniles	Obs.	80	40	25	9	3	.	0				
	Exp.		36.4	31.1	6.6	1.7	4.0	0.1	4	3.36	>.3	
Edmonton 2 Adults	Obs.	44	23	10	2	4	4	1				
	Exp.		20.5	12.3	1.8	2.0	6.8	0.6	4	4.17	>.2	
Edmonton 2 Juveniles	Obs.	74	34	25	1	3	9	2				
	Exp.		35.1	20.7	3.0	3.2	11.0	0.9	4	3.96	>.2	

Table XVII. Estimated allelic frequencies of antigenic types in various populations of deer mice

Deer Mouse Population	Number Tested	Estimated Allele Frequency		
		P _m ^A	P _m ^B	P _m ^C
Edm 1,1968	305	0.657	0.289	0.054
1969 Adults	138	0.678	0.279	0.043
1969 Juv.	80	0.675	0.288	0.037
Edm 2,1968	82	0.602	0.254	0.143
1969 Adults	44	0.682	0.205	0.113
1969 Juv.	74	0.689	0.203	0.108
Edm 3,1968	100	0.688	0.237	0.075
1969 Adults	46	0.663	0.283	0.054
1969 Juv.	41	0.671	0.256	0.073
British Columbia,1969	30	0.483	0.417	0.100
N. W. T.,1969	119	0.592	0.399	0.009
Montana,1970	53	0.368	0.189	0.443
Nisku 1,1970	70	0.507	0.214	0.278
Nisku 2,1970	118	0.533	0.338	0.127

Estimated allele frequency based on phenotypes observed.

DISCUSSION

The Pm blood group system was originally described as a simple two allele system (Rasmussen, 1961) and has now been expanded into a complex system with four and possibly six alleles. This adds the deer mouse to a growing list of vertebrates which have been shown to possess at least one multiple allele complex blood group system. Snell (1968) suggests that it is probably typical to find in each species of vertebrates or at least higher vertebrates, at least one extraordinarily complex blood group and/or histocompatibility locus. Several species (chickens, mice, rats) have shown that a single complex locus determines both the blood group antigens and histocompatibility antigens of a particular system. Thus it would not seem unusual if the Pm locus proved also to be a histocompatibility locus. If this proved to be the case then the deer mouse would provide an additional species for histocompatibility studies.

The study of complex systems determining blood group or histocompatibility antigens may take many approaches. The study may involve research into the functional properties of the products determined by the alleles of the locus, e.g., enzyme action, protein synthesis, membrane structures, or may involve research which deals with the antigenic properties *per se* of the system, e.g., maternal-fetal interactions, autoimmune disease. However, a very interesting property of all of the major complex blood group loci is the considerable polymorphism of alleles occurring at each locus and the relative stability of such polymorphisms, particularly as evidenced by general persistence of the polymorphism through several generations of inbreeding (Shultz and Briles, 1953; Cohen, 1962a).

These facts in themselves offer an interesting area for research.

Very little information is available concerning the mechanisms which result in extensive genetic polymorphisms. Snell (1968) and Palm (1970) have outlined several of the possible mechanisms which might contribute to the maintenance of stable polymorphisms of alleles determining cell membranes. These possibilities include selective advantage of the heterozygote, maternal-fetal interactions, selective membrane permeability or membrane attachment sites for viral or bacterial pathogens, and linkage of antigen determining loci to semi-lethal genes. The example of the last point was specifically the linkage of the H-2 locus to the T locus.

The Pm locus could prove interesting in studies on the mechanisms of maintaining genetic polymorphism at complex blood group loci since these species can be examined in many diverse natural populations as well as in the laboratory. Furthermore, the segregation distortion associated with AB females could prove to be associated in part with a mechanism which is responsible for ensuring polymorphism at the Pm locus. Segregation distortion as such has been observed in other species at certain loci, particularly the T locus in *Mus* (Dunn, 1964), and the linkage of this locus to the H-2 locus has been suggested as a possible mechanism for maintenance of polymorphism at that locus (Snell, 1968).

Since there is the strong possibility that the mechanism for determining the stable polymorphism of a complex blood group system may prove intrinsic to the animal, that is, unaffected by environment, it would seem evident that this mechanism should be carefully considered before the use of blood group antigens as markers can lead to precise answers about population structure.

Perhaps the final point worth mentioning again is the variation of allele frequency in different populations and the cross-reactivity of

anti-A with a closely related species, which indicates that further analysis of the Pm locus could prove valuable to the evolutionary biologist.

SUMMARY

1. A series of isoimmunization programs in deer mice (*Peromyscus maniculatus*) resulted in the production of four specificities of antisera. These antisera detected the presence of four antigens, A, B, C, and X, present on deer mouse erythrocytes.
2. Inheritance data showed that A, B, and C antigens were controlled by three codominant alleles at one autosomal locus. In addition, this data showed that AB females transmitted more A than B alleles to their offspring. No explanation could sufficiently account for this occurrence.
3. The X antigen was shown not to be an alternate antigen to A, B, or C, but was found associated with all B positive cells, certain C positive cells and rarely with A cells. Breeding data indicated that in crosses involving ACX mice X segregated with the C. Anti-X antiserum was found to be comprised of antibodies directed against at least two antigenic determinants. One antigenic component (X'') was specific to CX positive cells and the other component (X') was present on these cells and additionally on all B positive cells. A complex locus with at least four alleles was suggested to account for these findings. Two of these alleles determined more than one antigenic specificity, Pm^B determined antigenic specificity B and X', Pm^D determined antigenic specificity C, X', and X''.
4. A sample of Rasmussen's anti-B and typing results of mice from the Michigan laboratories indicated that the system described in this study was concerned with the Pm blood group locus. On the basis of certain typing reactions at these colonies two more alleles were hypothesized for the Pm locus.
5. Blood typing of various populations indicated that allele frequencies

generally remained consistent between parents and offspring, but variation in allele frequency did occur between areas.

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